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Identification and gene mapping of a narrow and upper-albino leaf mutant in rice (*Oryza sativa* L.)

WANG Feng, TANG YanQiang, MIAO RunLong, XU FangFang, LIN TingTing, HE GuangHua & SANG XianChun*

Rice Research Institute, Chongqing Key Laboratory of Application and Safety Control of Genetically Modified Crops, Engineering Research Center of South Upland Agriculture, Ministry of Education, Southwest University, Chongqing 400716, China

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The leaf blade consists of color and shape traits. Studies of leaf-blade development are important for improvement of rice yield and quality because it is an essential organ for photosynthesis. A narrow and upper-albino leaf mutant (*nul1*) was identified from among progeny of the *indica* restorer line Jinhui10 raised from seeds treated with ethyl methane sulfonate. Under field conditions, the mutant displayed narrow and upper-albino leaf blades with significantly decreased photosynthetic pigment contents throughout their development. The narrow-leaf trait is caused by a decreased number of small veins. In contrast to the wild type, the growth period was extended by approximately 8 d and agronomic traits, such as effective panicle number, percentage seed set and 1000-grain weight, declined significantly in the *nul1* mutant. Genetic analysis suggested that the narrow and upper-albino leaf characteristics showed coseparation and were controlled by one recessive gene. The *Nul1* gene was mapped onto chromosome 7 between the Indel marker Ind07-1 and the Simple Sequence Repeat marker RM21637. The physical distance between the markers was 75 kb and eight genes were annotated in this region based on the rice Nipponbare genome sequence. These results provide a foundation for cloning and function analysis of *Nul1*.

rice, *Oryza sativa*, narrow leaf, albino, gene mapping

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Rice (*Oryza sativa* L.) is a monocotyledonous plant and one of the most important food crops in the world. Rice is a staple food for more than half of the world's population. Leaves are the major photosynthetic organ. The development of leaf color, size, and shape can directly or indirectly influence the proportion of sunlight energy utilized and consequently have an important effect on yield and grain quality of cultivated rice. Therefore, leaf differentiation and development has received considerable attention in the breeding of rice ideotypes. Generally, leaf traits are classified into two groups in studies of leaf development: leaf color and leaf shape.

Leaf-color mutants mainly display an abnormal color such as abnormal whiting, etiolation, and striping [1]. At present, more than 90 leaf-color mutants have been identified in rice, and the responsible genes cloned from these mutants have been shown to function in, for example, chlorophyll synthesis and degradation, chloroplast growth, and the programmed cell death process [2–7]. Therefore, leaf-color mutants are the best material for investigation of the chlorophyll metabolic pathway, chloroplast development and gene regulation and the photosynthesis system [8,9].

Studies of leaf-shape development mainly focus on leaf rolling and size. Several rolled-leaf genes have been cloned and some breakthroughs in this regard have been achieved in rice recently [10–13]. However, few narrow-leaf traits have been studied because of the lack of functional deficiency mutants. At present, only seven narrow-leaf mutants are reported to be controlled by a single gene (*nal1*, *nal2*, *nal3*, *nal4*, *nal5*, *nal6* and *nal7*), of which *Nal1* and *Nal7*

*Corresponding author (email: sangxianchun@163.com)

have been isolated but the molecular mechanisms are still unclear [14–18]. Creation and identification of novel narrow-leaf mutants is important in order to isolate regulatory genes by a map-based cloning strategy and to further understand the mechanisms of rice leaf-shape development.

Previous studies on rice leaf development focused on a single character, e.g. color or shape, and no double-trait mutations were detected. In the present study, a narrow and upper-albino leaf mutant (*nul1*) was obtained from among progeny of the *indica* restorer line Jinhui10 raised from seeds treated with ethyl methane sulfonate. From a cross with the *japonica* cultivar Nipponbare, 4378 plants of F_2 lines exhibited both narrow-leaf and albino phenomena, which indicated that the two characters may show coseparation. The mutant is temporarily named *nul1* (narrow and upper-albino leaf 1). In this paper, we present the results of studying this specific mutant. These results provide a foundation for future cloning and function analysis of the regulatory gene.

1 Materials and methods

1.1 Plant materials

The narrow and upper-albino mutant (*nul1*) was obtained from among progeny of the *indica* restorer line Jinhui10 grown from seeds treated with ethyl methane sulfonate. Over the course of six generations, the mutation phenotype was genetically stable.

1.2 Measurement of agronomic traits

On the basis of leaf age, we investigated leaf width of the mutant and the wild type throughout the growth period. The length and width of the first, second and third leaves in the efflorescence were measured. The numbers of large and small veins were counted. In addition, the following agronomic traits were measured: plant height, number of effective panicles, percentage seed set, seed length and width, and 1000-grain weight.

1.3 Measurement of chloroplast pigment content

The first, second and third leaves of the *nul1* mutant and the wild type, respectively, were collected from plants at the heading stage at 8:30 a.m. to 9:00 a.m. in the efflorescence. Measurement of the content of chloroplast pigments (chlorophyll *a*, chlorophyll *b*, and carotenoid) followed previously described methods [19,20].

1.4 Construction of F_2 segregation population

A cross between the *nul1* mutant, as the male parent, and Nipponbare, as the female parent, was performed at the Rice Research Institute, Southwest University, Xiema, Chongqing, in summer 2009. The F_1 plants were grown in Hainan Province in autumn of the same year. The F_2 population was raised at the Rice Research Institute, Southwest University, in 2010.

1.5 DNA extraction

DNA of both parents and the gene pool was extracted using the CTAB method [21]. DNA of each individual of the F_2 segregation population was extracted using an alkaline-heating method [22].

1.6 SSR analysis

Ten randomly selected wild-type individuals and 10 mutant plants in the F_2 group were selected, equal quantities of genomic DNA were extracted to produce wild-type and mutant bulked DNAs, which were analyzed for markers polymorphic between the parents of the mapping population with 400 SSR primer pairs that were equally distributed on the rice genome. The potential linked markers were selected and verified in the F_2 mutant individuals. The selected SSR primer sequences corresponded to previously published RM SSR markers. DNA fragments were amplified in a 12.5-μL reaction volume that contained 1.25 μL 10× PCR reaction buffer, 0.65 μL $MgCl_2$ (2.5 mmol/L), 0.5 μL dNTPs (2.5 mmol/L), 8.0 μL ddH_2O, 1.0 μL of each primer (10 μmol/L), 1.0 μL DNA template, and 0.5 U TaKaRa *Taq* polymerase. Programmed for an initial 5 min at 94°C, then followed by 35 cycles for 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, and finally 10 min at 72°C. The PCR products were separated on 10% non-denatured PAGE gels and stained with silver nitrate solution [23].

1.7 Genetic mapping

The genotypes of Nipponbare, the *nul1* mutant and F_1 plants were scored as 'A', 'B' and 'H'. Linkage analysis was conducted with MAPMAKER 3.0, and the genetic distance (cM) was calculated with Kosambi function.

1.8 Fine mapping

On the basis of the initial mapping results and the published whole genome sequences of 93-11 and Nipponbare, an Indel marker in the *Nul1* locus region was designed using NTI vector 11.0 and Primer Premier 5.0.

2 Results

2.1 Phenotype of the *nul1* mutant

Under the natural field conditions, the *nul1* mutant exhibited an abnormal leaf color and leaf shape compared with the wild type (Figure 1(a) and (b)). The width of the leaves was

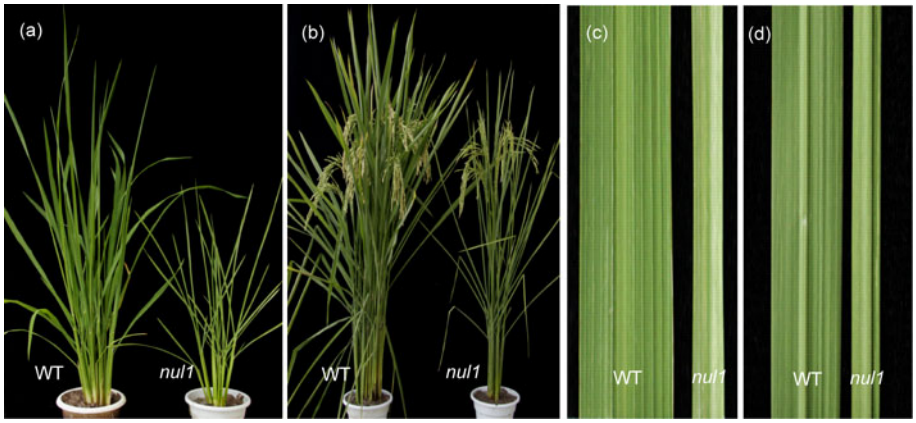

Figure 1 Phenotype of the *nul1* mutant and wild type (WT).

significantly reduced in the mutant throughout plant development (Figure 2). For example, at maturity, the leaf width of the first leaf to the third leaf in the mutant was only 47.42%, 54.55% and 49.35%, respectively, those of the wild type, whereas the length of these leaves showed no significant difference. Compared with the wild type, leaves of the *nul1* mutant contained a similar number of large veins but fewer small veins, the total number of small veins was only 60.32% that of the wild type, and the number of small veins between two adjacent large veins was 59.32% that of the wild type (Table 1). This should be the main causation of narrow leaf in the *nul1* mutant.

The *nul1* mutant also developed irregularly albino leaves but, interestingly, the albino trait was only apparent on the adaxial (upper) surface (Figure 1(c)) and not on the abaxial (lower) surface (Figure 1(d)) of the leaf blade. Photosynthetic pigment contents of the first, second and third upper leaves were measured at the heading stage. The chlorophyll *a*, chlorophyll *b*, and carotenoid contents were reduced, compared to the pigment contents of the flag leaf, which were only 58.87%, 53.68% and 66.07% the levels of the wild type (Figure 3).

Most of the agronomic traits investigated in the *nul1* mutant were significantly different from those of the wild type (Table 2). Grown under the same conditions, the *nul1* mutant exhibited a dwarf phenotype at the seedling stage but showed a similar height at maturity compared with the wild type. The heading date was delayed by about 8 d in the mutant and the number of effective panicles decreased remarkably by contrast with those of the wild type. The percentage seed set and 1000-grain weight were 56.47% and 74.30% those of the wild type; these differences were highly significant. However, seed length and width did not differ significantly.

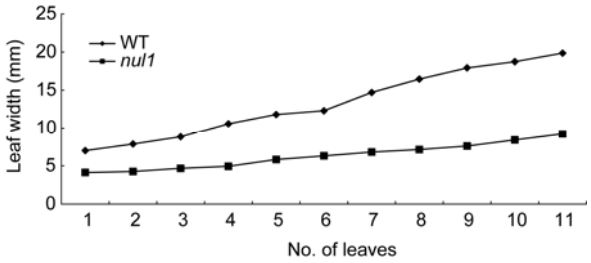

Figure 2 Leaf width of the *nul1* mutant and wild type throughout plant development. Leaf Nos. 1 to 11 represent the fourth leaf to the fourteenth leaf, respectively.

Table 1 Leaf phenotypic traits in the *nul1* mutant and wild type[a)]

	Length of flag leaf (cm)	Width of flag leaf (mm)	Length of second leaf (cm)	Width of second leaf (mm)	Length of third leaf (cm)	Width of third leaf (mm)	Number of large veins	Number of small veins	Number of small veins between two adjacent large veins
nul1	27.60±3.31*	9.20±0.79**	46.10±5.45*	8.40±0.84**	59.40±4.93	7.6±0.96**	8.66±0.57	26.3±1.52**	3.5±0.50**
WT	35±4.08	19.40±1.17	53.50±4.62	15.40±2.95	55.90±4.56	15.40±2.95	8.80±0.71	43.60±1.31	5.90±0.50

a) *, ** Significant at the 0.05 and 0.01 levels, respectively, with Student's *t*-test.

Table 2 Agronomic traits in the *nul1* mutant and wild type[a)]

	Plant height (cm)	Number of effective panicles	Seed set (%)	Seed length (mm)	Seed width (mm)	1000-grain weight (g)
nul1	104.70±2.31	8.6±0.96*	48±0.02**	6.91±0.30	2.19±0.05	19.17**
WT	107.58	11.12	85	7.13±0.10	2.37±0.08	25.8

a) *, ** Significant at the 0.05 and 0.01 levels, respectively, with Student's *t*-test.

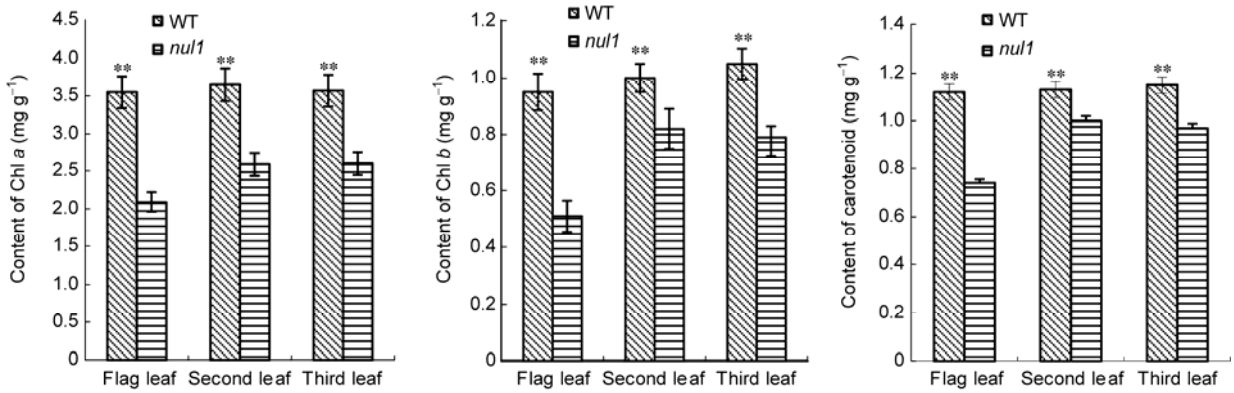

Figure 3 Photosynthetic pigment contents in the *nul1* mutant and wild type. ** Significant at the 0.01 level with Student's *t*-test.

2.2 Genetic analysis

The wild-type leaf phenotype was observed in the F_1 plants derived from the cross between the *nul1* mutant and Nipponbare. Two types of plants were present in the F_2 population: those with wild-type leaves, and those with narrow upper-albino leaves like the *nul1* mutant. The ratio of normal leaves to mutant-type leaves was 3325∶1053, which conformed to a 3∶1 ratio (χ^2=2.05 < $\chi^2_{0.05}$=3.84). These results suggested that the mutational traits of narrow leaves and upper-albino leaves showed coseparation and were controlled by one nuclear gene in the *nul1* mutant.

2.3 Gene mapping of the *Nul1* locus

A total of 1053 F_2 mutant plants derived from the cross between Nipponbare and the *nul1* mutant were used as a mapping population in accordance with a bulked segregant analysis strategy [24]. The SSR analysis showed that the *Nul1* locus was located between the SSR markers RM445 and RM3826 on chromosome 7; the genetic distances were 4 and 3.3 cM, respectively (Figure 4).

New SSR and Indel markers within the primary restricted region of the *Nul1* locus were developed and six markers that showed polymorphisms between the *nul1* mutant and Nipponbare were utilized to analyze 1053 F_2 mutant individuals. The markers of RM21615, RM6394 and Ind07-1 (forward primer: 5′-ATGTAACCTACTTACTGCTGCG-3′, reverse primer: 5′-ATACAGCAACATGCACCATTG-3′) identified 15, 8 and 1 recombinants, respectively. Three additional markers (RM21637, RM21638 and RM21650) identified 1, 6 and 13 recombinants, which differed with the markers of RM21615, RM6394, Ind07-1. These results indicated that the *Nul1* locus was located between the Ind07-1 and RM21637 markers and the physical distance was about 75 kb on the basis of the Nipponbare genome sequence (Figure 5).

Eight genes were annotated within the restricted region on the basis of information in the Gramene database (http://www.gramene.org/). The genes comprised two expressed proteins, two hypothetical proteins, one CHR4/MI-2-LIKE, one peptide-nasparagine amidase, one MYB family transcription factor, and one leucine-rich repeat receptor protein kinase EXS precursor. This finding provides a solid foundation for cloning and function analysis of the *Nul1* gene in the future.

3 Discussion

Mutations of color and shape are both obvious characters which alter leaf blade morphology in *Oryza sativa*. Leaf color can be influenced by environment and genetic factors; the leaf color phenotype is complex and generally involves chlorophyll biosynthesis and metabolism, hemachrome biosynthesis and metabolism, and chloroplast gene-encoded proteins [25]. Currently, at least 90 leaf-color mutants have been identified in rice. Regulatory genes or loci are located on every chromosome except chromosome 12. More than 20 rice mutants exhibit a leaf blade with albino or white-stripe characters. Compared with previously known mutants,

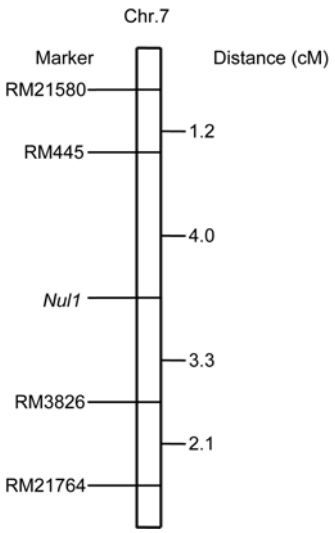

Figure 4 Location of the *Nul1* locus on chromosome 7.

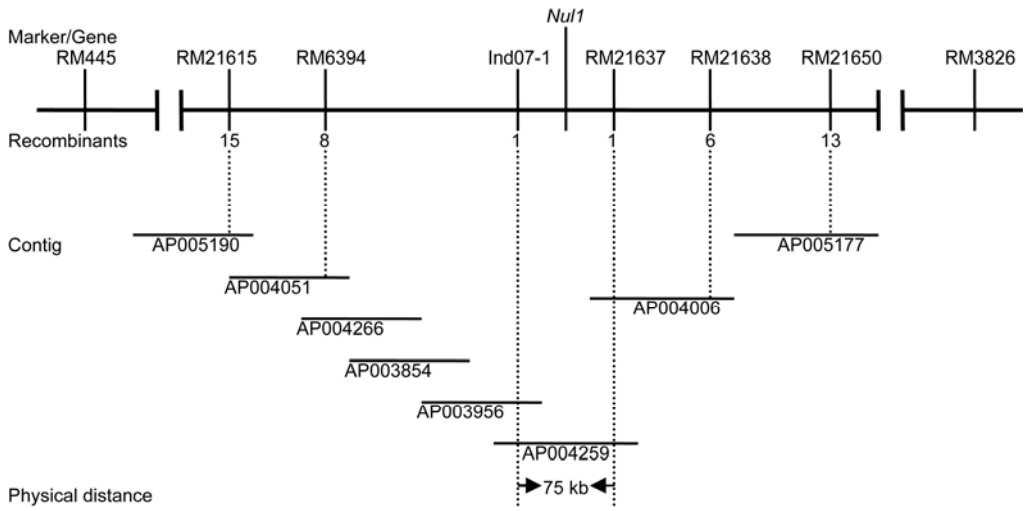

Figure 5 Fine mapping of the *Nul1* locus.

the *nul1* mutant displays distinctive leaf blades throughout the life of the plant. Specifically, the leaf blade only shows the albino trait on the adaxial surface and not on the abaxial surface.

The other crucial morphological abnormality in the *nul1* mutant is that the leaf blades are highly significantly narrower than the wild-type leaves. Leaf development is a complicated procedure that involves processes such as phyllome initiation and leaf-polar construction. The growth of middle-margin axes is generally accepted to be involved in narrow-leaf traits, but the regulatory genes and corresponding interaction network are still unknown in rice [26,27]. Only three such genes have been cloned at present. The *Nd1* gene encodes the cellulose synthase-like protein D4 (*OsCslD4*), which regulates leaf morphogenesis and vegetative development by affecting cell wall development; three allelic mutants have been identified and show similar narrow and rolled leaf characters [28,29]. The *Nal1* gene encodes a unique plant protein of unknown function and influences organ development by altering the polar transportation of auxin; the reported mutant shows dwarfism and a narrow-leaf phenotype [17]. The *nal7* mutant significantly decreased the IAA content in the narrow leaf blade, which suggested the wild-type gene *Nal7* encodes a flavin-containing monooxygenase that displays sequence homology with *YUCCA*, a gene involved in auxin biosynthesis [16]. Auxin has an important role in polar axis determination of the leaf blade and either polar transportation or biosynthesis of auxin may influence leaf development, including the narrow leaf phenotype. Discovery and identification of the *nul1* mutant should enrich our knowledge of polar development of the leaf blade.

Leucine-rich repeat receptor-like protein kinases (LRR-RLKs) localized at the plasma membrane is the largest family of receptor kinases in plants. At least 216 members of the family have been identified in *Arabidopsis* and more than 300 in rice. LRR-RLKs are important in the transduction of various plant environmental and developmental signals [30]. MYB, a plant transcription factor family, can regulate plant development and physiological metabolism by altering the transcription level of other genes. Many MYB members have been identified in plant, for example, 198 in *Arabidopsis*, about 200 in cotton, and more than 80 in maize. Some MYB transcription factors regulate secondary metabolism, hormone biosynthesis and metabolism, response to environmental factors, and plant developmental processes such as cell differentiation, cell cycle and morphogenesis of leaf shape. Recently, a MYB transcription factor was shown to assist in the accumulation of plant pigments, which was crucial for color formation in the leaf blade and flower [31]. There are eight genes, including a MYB transcription factor and a LRR-RLK, in the restricted region of the *Nul1* locus. The MYB transcription factor was sequenced but on difference between the wild type and the *nul1* mutant suggests that the *nul1* mutant is controlled by one novel type of gene.

The *nul1* mutant reported here shows a leaf blade remodeled in both color and shape. The mutant leaf blades exhibit the albino trait only on the adaxial surface and are highly significantly narrower than the wild type throughout plant development. The *nul1* mutant is indicated to be a novel type of leaf trait mutant. Further studies of this mutant will enrich our understanding of the development of both color and shape of the leaf blade.

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